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Colletotrichum nymphaeae var. *entomophilum* var. nov. a natural enemy of the citrus scale insect, *Praelongorthezia praelonga* (Hemiptera: Ortheziidae)

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ABSTRACT: The citrus scale insect *Praelongorthezia praelonga* (Douglas), a major pest of citrus and other economically important crops, has only two commonly documented natural enemies: an entomopathogenic strain of the fungus *Colletotrichum nymphaeae* (Pass.) Aa and several parasitoids. The entomopathogenic strain of *C. nymphaeae*, formerly recognized under the synonym *C. gloeosporioides* f. sp. *ortheziidae*, is under development for commercial application as a biological control agent in citrus in Brazil-the top exporter of citrus globally. The synonymy of *C. gloeosporioides* f. sp. *ortheziidae* with *C. nymphaeae* remains based on limited DNA sequence data and without morphological study. To qualify for future approval as a biological control agent by federal agencies in Brazil and the European Union, the circumscription of a microorganism must be explicit and without ambiguities. Herein, through morphological study and phylogenetic analysis of five DNA regions we clarify the circumscription and affinity of entomopathogenic *C. nymphaeae* and describe it as a new variety.

Keywords: Biological control, *Colletotrichum* (Sordariomycetes: Glomerellaceae), entomopathogen

Introduction

Colletotrichum Corda (Ascomycota: Sordariomycetes: Glomerellaceae) is a cosmopolitan and speciose genus of fungi comprised largely of plant symbionts (Damm et al., 2012; Hanlin, 1998). The host range within the genus is broad with both pathogenic and endophytic species reported from all major lineages of land plants (MacKenzie et al., 2009; Manamgoda et al., 2013; Photita et al., 2005). Two *Colletotrichum* taxa are entomopathogenic: *C. fiorinae* and *C. nymphaeae*. *Colletotrichum fiorinae* was described from the hemlock scale insect *Fiorna externa* Ferris; however, *C. nymphaeae* is a widespread plant pathogen in which entomopathogenicity is an exception and limited to strains isolated from the citrus scale insect *Praelongorthezia praelonga* (Douglas) (syn. *Orthezia praelonga* Douglas) (Hemiptera: Ortheziidae) (Marcelino et al., 2008).

Entomopathogenic *C. nymphaeae* is of particular interest because the citrus scale insect (*P. praelonga*), its primary host, is a serious pest of *Citrus* spp. and other major economic plants such as coffee, figs and ornamentals (Garcia-Roa, 1995). Citrus scale is considered a pest wherever it is found because of its elevated reproductive rate and highly polyphagous nature (Kondo et al., 2013). The regularly occurring natural enemies of citrus scale are currently limited to *C. nymphaeae* and several parasitoids (Kondo et al., 2013; Ramos et al., 2018). Studies on entomopathogenic *C. nymphaeae* have focused on its utility and development as a biological control agent of *P. praelonga* in citrus production (Teixeira et al., 2001; Teixeira et al., 2004). Natural outbreaks of the fungus with high mortality of citrus scale are observed in conventional citrus groves; outbreaks appear to be density dependent with increased prevalence during rainy and warm weather (Mascarin et al., 2016).

In Brazil, *C. nymphaeae* on citrus scale is colloquially known as the salmão (salmon) fungus-a reference to the characteristic salmon pink color of the conidial masses on infected insects. The salmão fungus, previously *C. gloeosporioides* f. sp. *ortheziidae*, was transferred to *C. nymphaeae* based on a single locus and without a comparative morphological study (Damm et al., 2012; Marcelino et al., 2008). The designation *formae speciales* (f. sp.) was dropped by this transfer; therefore, entomopathogenic isolates of *C. nymphaeae* are no longer distinguished by name from its plant pathogenic conspecifics. Because of its potential for biological control, the taxonomy and affinity of the salmão fungus require further study. In this study, through morphological and molecular studies, we take a polyphasic approach to clarifying the taxonomy of this important natural enemy of the citrus scale insect.

Materials and Methods

Fungal isolation and preservation

Two isolates of *Colletotrichum* sp., collected from the citrus scale insect, were purified *in vitro* on potato-dextrose-agar and subsequently deposited in the Laboratório de Patologia e Controle Microbiano, Departamento de Entomologia e Acarologia of the Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo (USP/ESALQ). Isolate ESALQ 1393 was collected in Limeira, state of São Paulo, 8 Feb 2007, *Sylvio Baggio s.n.*, and isolate ESALQ 1368 from Cordeirópolis, state of São Paulo, 13 July 2005, *Luiz Fernando Padulla s.n.* Both isolates were preserved in a lyophilized state and in sterile 10 % glycerol at –80 °C as metabolically inactive strains.

Morphological study

Morphological study and descriptions were made from observations of fungal structures mounted in wa-

ter on a glass slide. Measurements and light photomicrographs were taken using an Olympus AX70 Provis compound light microscope and Cell ^A analysis image processing software as well as an Olympus SZX16 dissecting microscope. Herbarium acronyms followed those of the Index Herbariorum (Thiers, 2018). Morphological characters were recorded from cultures grown on Sabouraud dextrose agar (SDA), oatmeal agar (OA) (Crous et al., 2009), and synthetic nutrient-poor agar medium (SNA) (Nirenberg, 1976) and maintained at 22 °C with a light regime of 18 h of darkness and 6 h of light. Figures were assembled with Adobe Photoshop CS4.

Molecular study

Genomic DNA was extracted from *C. nymphaeae* isolates from infected *P. praelonga* individuals collected from citrus orchards in Brazil. One isolate, ARSEF 4360, was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) and ESALQ 1393 was obtained from the culture collection of the Laboratório de Patologia e Controle Microbiano, Universidade de São Paulo (USP/ESALQ). DNA from ESALQ 1393 and for ARSEF 4360 were extracted as described in Mascarin et al. (2016); genomic DNA was obtained by grinding the mycelium with a plastic pestle inside a 1.5 mL Eppendorf tube. DNA was then isolated using a commercial plant DNA extraction kit using the standard protocol and eluted with 100 µL sterile deionized water.

Three loci were amplified: the entire nrDNA ITS region (ITS1-5.8S-ITS2), actin (ACT) and chitin synthase (CHS-1). The entire nrDNA ITS region (ITS1-5.8S-ITS2) was amplified using primer pairs ITS1F (Gardes and Bruns, 1993) + ITS4 (White et al., 1990), ACT and CHS-1 were amplified and sequenced using the primer pairs ACT-512F + ACT-783R and CHS-354R + CHS-79F (Carbone and Kohn, 1999), respectively. Primers and primer sequences are listed in Table 1. PCR was performed under the following conditions for ITS: step 1) 1 min at 95 °C, 2) 45 s at 95 °C, 3) 40 s, 50.8 °C, 4) 90 s at 72 °C, 5) return to step 2 34 times, 6) a final step of 10 min at 72 °C; for both ACT and CHS-1: step 1) 3 min at 95 °C, 2) 15 s at 95 °C, 3) 20 s at 58 °C, 4) 1 min at 72 °C, 5) return to step 2 34 times, 6) final step of 5 min at 72 °C. Samples were kept at 10 °C until electrophoresis was performed on a 1 % agarose TAE gel and visualized under UV light. PCR products were cleaned using a PCR purification kit and sent for sequencing. Sequences were

assembled with Sequencher v. 5.3 and aligned manually in MEGA5 (Tamura et al., 2011).

Using the newly obtained sequences as well as sequences from GenBank, including those generated by Damm et al. (2012), five datasets were produced: ITS and TUB2 datasets each having 77 sequences, and glyceraldehyde-3-phosphate dehydrogenase (GADPH), CHS-1 and ACT datasets each having 76 sequences. A total of three strains of *C. nymphaeae* isolated from *P. praelonga* were included in the molecular study: ESALQ 1368, ESALQ 1393, ARSEF 4360; however, only the last two, for which multiple loci were obtained, were included in the combined phylogenetic analysis. The taxa and isolates used in the study are shown in Table 2.

The individual ITS, GADPH, CHS-1, ACT, TUB2 matrices were exported in NEXUS format and converted to a combined data file in PAUP* v. 4.0.10b. The individual datasets were analyzed by maximum parsimony using a heuristic search with random addition sequence, TBR swapping and 1,000 heuristic replicates, saving no more than ten best trees per replicate, followed by a final search of saved trees. The phylograms of each dataset were visually inspected for topological congruence and then combined into a single dataset. The combined dataset was bootstrapped (2,000 replicates) using the same maximum parsimony search parameters. Following Damm et al. (2012), *C. orchidophilum* was used as the outgroup for the *C. acutatum* complex. A final phylogram with added bootstrap values was prepared using the Adobe Illustrator Professional. The combined dataset was deposited in TreeBase and can be accessed at <http://purl.org/phylo/treebase/phyloids/study/TB2:S18116>

Results

Molecular study

The combined ITS + GADPH + CHS-1 + ACT + TUB2 dataset included 1,661 characters of which 1,233 were constant, 50 were variable but not parsimony-informative, and 378 were parsimony-informative. A total of 600 equally most-parsimonious trees of 432 steps were recovered: a phylogram of one of these trees is shown in Figure 1. Isolates from the citrus scale insect are in a well-supported *C. nymphaeae* clade [97 % bootstrap support (bs)] (Figure 1). The combined dataset recovered two major clades for *C. nymphaeae* with respective bootstrap support values of 86 % and 89 %. The citrus

Table 1 – Primer sequences and references for loci amplified in this study: ACT = actin, CHS-1 = chitin synthase 1 and ITS = Internal transcribed spacer (ITS) situated between the small-subunit ribosomal RNA.

Locus	Primers	Sequence (5'–3')	Reference
ACT	ACT-512F	ATG TGC AAG GCC GGT TTC GC	Carbone and Kohn, 1999
	ACT-783R	TAC GAG TCC TTC TGG CCC AT	Carbone and Kohn, 1999
CHS-1	CHS-354R	TGG AAG AAC CAT CTG TGA GAG TTG	Carbone and Kohn, 1999
	CHS-79F	TGG GGC AAG GAT GCT TGG AAG AAG	Carbone and Kohn, 1999
ITS	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Brunes, 1993
	ITS4	TCC TCC GCT TAT TGA TAT GC	White et al., 1990

Table 2 – Host information, geographical origin and ITS, ACT, CHS-1, GADPH and TUB2 GenBank accession numbers of the taxa/strains included in the molecular study. Types are designated by an asterisk. Insect hosts are underlined.

Species	Isolate/Strain	Host/substrate	Geographic origin	ITS	ACT	CHS-1	GADPH	TUB2
<i>C. acerbum</i>	CBS 128530*	<i>Malus domestica</i>	New Zealand	JQ948459	JQ949780	JQ949120	JQ948790	JQ950110
<i>C. acutatum</i>	CBS 144.29	<i>Capsicum annuum</i>	Sri Lanka	JQ948401	JQ949722	JQ949062	JQ948732	JQ950052
	CBS 112996*	<i>Carica papaya</i>	Australia	JQ005776	JQ005839	JQ005797	JQ948677	JQ005860
	CBS 979.69	<i>Coffea arabica</i>	Kenya	JQ948400	JQ949721	JQ949061	JQ948731	JQ950051
	CBS 370.73	<i>Pinus radiata</i>	New Zealand	JQ948351	JQ949672	JQ949012	JQ948682	JQ950002
	IMI 336479	<i>Pistacia vera</i>	Australia	JQ948367	JQ949688	JQ949028	JQ948698	JQ950018
	CBS 113006	<i>Protea cynaroides</i>	South Africa	JQ948390	JQ949711	JQ949051	JQ948721	JQ950041
<i>C. australe</i>	CBS 116478*	<i>Trachycarpus fortunei</i>	South Africa	JQ948455	JQ949776	JQ949116	JQ948786	JQ950106
	CBS 131325	<i>Hakea</i> sp.	Australia	JQ948456	JQ949777	JQ949117	JQ948787	JQ950107
<i>C. brisbanense</i>	CBS 292.67*	<i>Capsicum annuum</i>	Australia	JQ948291	JQ949612	JQ948952	JQ948621	JQ949942
<i>C. cosmi</i>	CBS 853.73*	<i>Cosmos</i> sp.	Netherlands	JQ948274	JQ949595	JQ948935	JQ948604	JQ949925
<i>C. costaricense</i>	CBS 330.75*	<i>Coffea arabica</i>	Costa Rica	JQ948180	JQ949501	JQ948841	JQ948510	JQ949831
	CBS 211.78	<i>Coffea</i> sp.	Costa Rica	JQ948181	JQ949502	JQ948842	JQ948511	JQ949832
<i>C. cuscatae</i>	IMI 304802*	<i>Cuscuta</i> sp.	Dominica	JQ948195	JQ949516	JQ948856	JQ948525	JQ949846
<i>C. fioriniae</i>	CBS 125396	<i>Malus domestica</i>	USA	JQ948299	JQ949620	JQ948960	JQ948629	JQ949950
	IMI 345578	<i>Fragaria</i> × <i>ananassa</i>	New Zealand	JQ948330	JQ949655	JQ948995	JQ948660	JQ949981
	CBS 126523	<i>Berberis</i> sp.	Netherlands	JQ948322	JQ949643	JQ948983	JQ948652	JQ949973
	CBS 786.86	<i>Malus sylvestris</i>	Italy	JQ948303	JQ949624	JQ948964	JQ948633	JQ949954
	CBS 112995	<i>Malus domestica</i>	USA	JQ948298	JQ949619	JQ949289	JQ948628	JQ949949
	CBS 981.69	<i>Coffea arabica</i>	Angola	JQ948327	JQ949648	JQ948988	JQ948657	JQ949978
	CBS 200.35	<i>Rubus</i> sp.	USA	JQ948293	JQ949614	JQ948954	JQ948623	JQ949944
	CBS 490.92	<i>Solanum lycopersicum</i>	New Zealand	JQ948326	JQ949647	JQ948987	JQ948656	JQ949977
	CBS 129948	<i>Tulipa</i> sp.	UK	JQ948344	JQ949665	JQ949005	JQ948674	JQ949995
	CBS 119292	<i>Vaccinium</i> sp.	New Zealand	JQ948313	JQ949634	JQ948974	JQ948643	JQ949964
<i>C. godetiae</i>	CBS 133.44*	<i>Clarkia hybrida</i>	Denmark	JQ948402	JQ949723	JQ949063	JQ948733	JQ950053
	CBS 129934	<i>Prunus dulcis</i>	Israel	JQ948431	JQ949752	JQ949092	JQ948762	JQ950082
	CBS 862.70	<i>Sambucus nigra</i>	Netherlands	JQ948437	JQ949758	JQ949098	JQ948768	JQ950088
	CBS 127561	<i>Ugni molinae</i>	Chile	JQ948442	JQ949763	JQ949103	JQ948773	JQ950093
<i>C. guajavae</i>	IMI 350839*	<i>Psidium guajava</i>	India	JQ948270	JQ949591	JQ948931	JQ948600	JQ949921
<i>C. indonesiense</i>	CBS 127551*	<i>Eucalyptus</i> sp.	Indonesia	JQ948288	JQ949609	JQ948949	JQ948618	JQ949939
<i>C. johnstonii</i>	IMI 357027	<i>Citrus</i> sp.	New Zealand	JQ948443	JQ949764	JQ949104	JQ948774	JQ950094
	CBS 128532*	<i>Solanum lycopersicum</i>	New Zealand	JQ948444	JQ949765	JQ949105	JQ948775	JQ950095
<i>C. kinghornii</i>	CBS 198.35*	<i>Phormium</i> sp.	UK	JQ948454	JQ949775	JQ949115	JQ948785	JQ950105
<i>C. laticiphilum</i>	CBS 112989*	<i>Hevea brasiliensis</i>	India	JQ948289	JQ949610	JQ948950	JQ948619	JQ949940
	CBS 129827	<i>Hevea brasiliensis</i>	Colombia	JQ948290	JQ949611	JQ948951	JQ948620	JQ949941
<i>C. limetticola</i>	CBS 114.14*	<i>Citrus aurantifolia</i>	USA, Florida	JQ948193	JQ949514	JQ948854	JQ948523	JQ949844
<i>C. lupini</i>	CBS 129944	<i>Cinnamomum verum</i>	Portugal	JQ948178	JQ949499	JQ948839	JQ948508	JQ949829
	CBS 513.97	<i>Lupinus polyphyllus</i>	Costa Rica	JQ948157	JQ949478	JQ948818	JQ948487	JQ949808
	CBS 466.76	<i>Manihot utilissima</i>	Rwanda	JQ948160	JQ949481	JQ948821	JQ948490	JQ949811
<i>C. melonis</i>	CBS 159.84*	<i>Cucumis melo</i>	Brazil	JQ948194	JQ949515	JQ948855	JQ948524	JQ949845
<i>C. nymphaeae</i>	CBS 130.80	<i>Anemone</i> sp.	Italy	JQ948226	JQ949547	JQ948887	JQ948556	JQ949877
	IMI 379162	<i>Capsicum annuum</i>	Zimbabwe	JQ948218	JQ949539	JQ948879	JQ948548	JQ949869
	CBS 129945	<i>Olea europaea</i>	Portugal	JQ948201	JQ949548	JQ948888	JQ948531	JQ949852
	IMI 360386	<i>Pelargonium graveolens</i>	India	JQ948206	JQ949527	JQ948867	JQ948536	JQ949857
	ESALQ 1368	<i>Praelongorthezia praelonga</i>	Brazil	—	—	—	—	KJ509200
	ESALQ 1393*	<i>P. praelonga</i>	Brazil	MG547976	MG572798	MG572799	—	KJ509199
	ARSEF 4360	<i>P. praelonga</i>	Brazil	EF593371	MG572800	MG572801	EF593346	EF593327
	CBS 361.79	<i>Anemone coronaria</i>	Netherlands	JQ948248	JQ949569	JQ948909	JQ948578	JQ949899
	CBS 126377	<i>Fragaria</i> × <i>ananassa</i>	Netherlands	JQ948233	JQ949554	JQ948894	JQ948563	JQ949884
	CBS 112202	<i>Fragaria</i> sp.	Spain	JQ948234	JQ949555	JQ948895	JQ948564	JQ949885
<i>C. nymphaeae</i>	CBS 129926	Litter	Thailand	JQ948216	JQ949537	JQ948877	JQ948546	JQ949867
	CBS 516.78	<i>Nuphar luteum</i>	Netherlands	JQ948198	JQ949519	JQ948859	JQ948528	JQ949849
	CBS 515.78*	<i>Nymphaea alba</i>	Netherlands	JQ948197	JQ949518	JQ948858	JQ948527	JQ949848

Continue.

Table 2 – Continuation.

	CSL 455	<i>Photinia</i> sp.	UK	JQ948217	JQ949538	JQ948878	JQ948547	JQ949868
	EMA26	<i>Praelongorthezia praelonga</i>	Brazil	EF593372	—	—	EF593347	EF593328
	CBS 112992	<i>Protea magnifica</i>	South Africa	JQ948207	JQ949528	JQ948868	JQ948537	JQ949858
<i>C. orchidophilum</i>	CBS 631.80	<i>Ascocenda</i> sp.	USA	JQ948152	JQ949473	JQ948813	JQ948482	JQ949803
	CBS 632.80*	<i>Dendrobium</i> sp.	USA	JQ948151	JQ949472	JQ948812	JQ948481	JQ949802
<i>C. paxtonii</i>	CBS 502.97	<i>Musa nana</i>	West Indies	JQ948286	JQ949607	JQ948947	JQ948616	JQ949937
	IMI 165753*	<i>Musa</i> sp.	Saint Lucia	JQ948285	JQ949606	JQ948946	JQ948615	JQ949936
<i>C. phormii</i>	CBS 118191	<i>Phormium</i> sp.	South Africa	JQ948453	JQ949774	JQ949114	JQ948784	JQ950104
	CBS 118197	<i>Phormium</i> sp.	New Zealand	JQ948450	JQ949771	JQ949111	JQ948781	JQ950101
	CBS 483.82	<i>Phormium tenax</i>	New Zealand	JQ948451	JQ949772	JQ949112	JQ948782	JQ950102
<i>C. pyricola</i>	CBS 128531*	<i>Pyrus communis</i>	New Zealand	JQ948445	JQ949766	JQ949106	JQ948776	JQ950096
<i>C. rhombiforme</i>	CBS 129953 *	<i>Olea europaea</i>	Portugal	JQ948457	JQ949778	JQ949118	JQ948788	JQ950108
	CBS 131322	<i>Vaccinium macrocarpum</i>	USA	JQ948458	JQ949779	JQ949119	JQ948789	JQ950109
<i>C. salicis</i>	CBS 465.83	<i>Araucaria excelsa</i>	USA	JQ948468	JQ949789	JQ949129	JQ948799	JQ950119
	CBS 607.94*	<i>Salix</i> sp.	Netherlands	JQ948460	JQ949781	JQ949121	JQ948791	JQ950111
<i>C. scovillei</i>	CBS 126529*	<i>Capsicum</i> sp.	Indonesia	JQ948267	JQ949588	JQ948928	JQ948597	JQ949918
	CBS 120708	<i>Capsicum annuum</i>	Thailand	JQ948269	JQ949590	JQ948930	JQ948599	JQ949920
<i>C. simmondsii</i>	CBS 294.67	<i>Carica papaya</i>	Australia	JQ948277	JQ949598	JQ948938	JQ948607	JQ949928
	CBS 126524	<i>Cyclamen</i> sp.	Netherlands	JQ948281	JQ949602	JQ948942	JQ948611	JQ949932
<i>C. sloanei</i>	IMI 364297*	<i>Protea cynaroides</i>	Malaysia	JQ948287	JQ949608	JQ948948	JQ948617	JQ949938
<i>C. tamarilloi</i>	CBS 129814*	<i>Theobroma cacao</i>	Colombia	JQ948184	JQ949505	JQ948845	JQ948514	JQ949835
	CBS 129956	<i>Solanum betaceum</i>	Colombia	JQ948190	JQ949511	JQ948851	JQ948520	JQ949841
<i>C. walleri</i>	CBS 125472*	<i>Coffea</i> sp.	Vietnam	JQ948275	JQ949596	JQ948936	JQ948605	JQ949926
<i>C. sp.</i>	CBS 129810	<i>Solanum betaceum</i>	Colombia	JQ948179	JQ949500	JQ948840	JQ948509	JQ949830

scale insect isolates are in the same clade as isolate CBS 515.78, the type of *C. nymphaeae* designated as *C. nymphaeae* var. *nymphaeae* in Figure 1. In the beta-tubulin dataset, a single nucleotide mutation was present in all four entomopathogenic isolates of *C. nymphaeae*: this mutation is shared only by one other species, *C. australe*, which is also in the *C. acutatum* complex, but distantly related to *C. nymphaeae* (Table 3; Figure 1). Isolates of *C. nymphaeae* from the citrus scale insect differed from all other *C. nymphaeae* isolates by two nucleotide changes in the CHS-1 gene (Table 4). Insect pathogenic *C. nymphaeae*, colloquially referred to as the salmão fungus is described herein as a new variety of *C. nymphaeae*.

Taxonomy

Collectrichum nymphaeae var. *entomophilum*

A.A. Wynns & I. Delalibera, **var. nov.**

MycoBank 823559 Figure 2A-H

= *C. gloeosporioides* f. sp. *ortheziidae* (Marcelino et al., 2008)

Etymology: The varietal epithet, which means insect loving, indicates its predilection for insects.

Type: Limeira, state of São Paulo, isolated from a *Praelongorthezia praelonga* (Hemiptera: Ortheziidae) cadaver, 8 Feb 2007, *Silvio Baggio s.n.* Holotype, *ESA 142963* permanently preserved in a metabolically inactive state as a dried culture in microscope slides of *ESALQ 1393-H* deposited in the herbarium of Escola

Table 3 – Beta-tubulin gene sequence segment showing single nucleotide mutation present in all four isolates of *C. nymphaeae* var. *entomophilum* and shared by only one other taxon (*C. australe*) within the *C. acutatum* complex.

Taxon	Isolate	Beta-tubulin sequence
<i>C. nymphaeae</i>		
var. <i>entomophilum</i>	ARSEF 4360	TCAC-TCGTTCTCCAGTG
	EMA 26	TCAC-TCGTTCTCCAGTG
	ESALQ 1368	TCAC-TCGTTCTCCAGTG
	ESALQ 1393	TCAC-TCGTTCTCCAGTG
var. <i>nymphaeae</i>	IMI 360386	TCAC-TCGTCTCCAGTG
	CBS 515.78	TCAC-TCGTCTCCAGTG
<i>C. australe</i>		
	CBS 131325	TCTC-TTGTCTCCAGTG
	CBS 116478	TCTC-TTGTCTCCAGTG

Table 4 – CHS-1 gene nucleotide differences between *C. nymphaeae* var. *nymphaeae* and *C. nymphaeae* var. *entomophilum*.

Taxon	Isolate	Mutations and their position in the CHS-1 gene sequence	
<i>C. nymphaeae</i>		217	247
var. <i>entomophilum</i>	ARSEF 4360	C	T
	ESALQ 1393	C	T
var. <i>nymphaeae</i>	CBS 515.78	T	C
	IMI 360386	T	C
	CBS 130.80	T	C

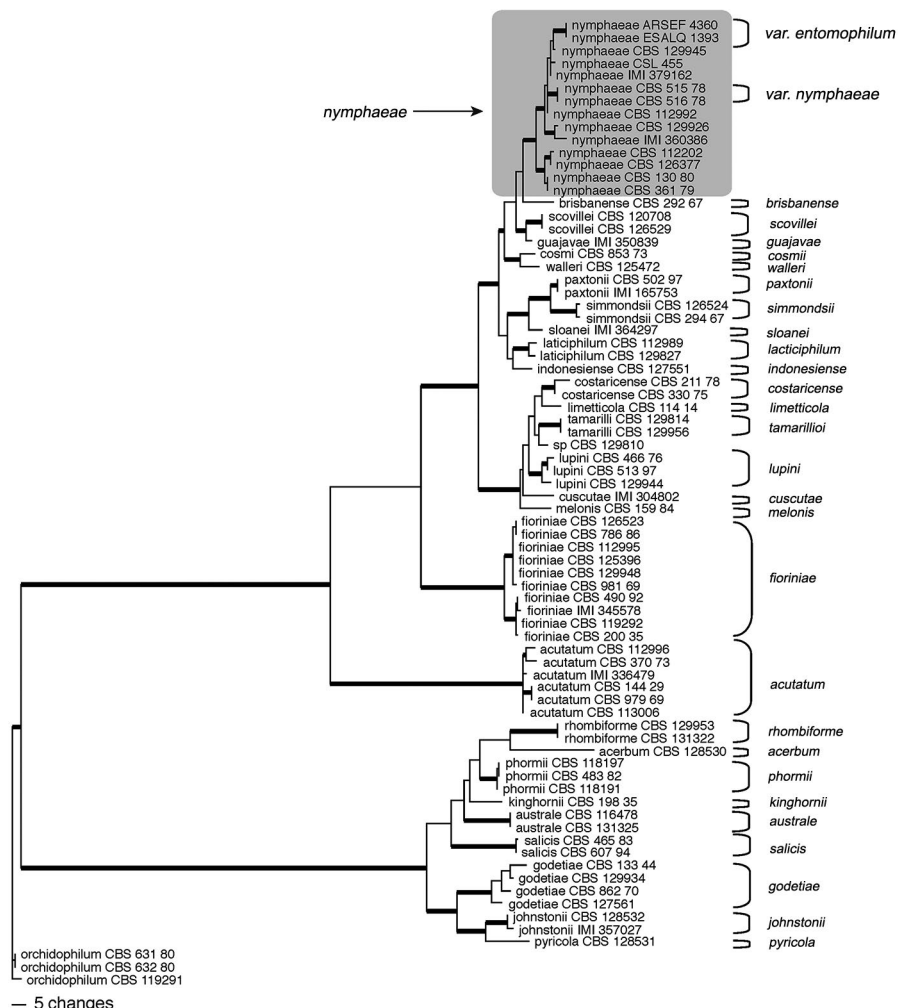


Figure 1 – One of 9,139 equally most-parsimonious phylograms from a maximum parsimony analysis of ITS + GADPH + CHS-1 + ACT + TUB2 combined sequence data from 73 isolates within the *Colletotrichum acutatum* species complex. Thickened branches lead to nodes with bootstrap support values $\geq 70\%$ (2000 replicates).

Superior de Agricultura “Luiz de Queiroz” from the Universidade de São Paulo (USP/ESALQ/ESA - <http://splink.cria.org.br/manager/detail?setlang=pt&resource=ESA>).

Ex-holotype cultures are stored in lyophilized inactive state as *ESALQ 1393-H* in the Collection of Entomopathogenic Fungi, Laboratório de Patologia e Controle Microbiano, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo (USP/ESALQ), Piracicaba, state of São Paulo, and as *CG1414* in the Invertebrate-Associated Fungal Collection of Embrapa (CG), Embrapa Genetic Resources and Biotechnology, DF-70770-970, Brazil (http://www.wfcc.info/ccinfo/collection/by_id/712).

Colonies on OA 10.5 cm at seven days, a low cottony mycelium with concentric alternating white, salmon and grey rings (Figure 2A); salmon color from numerous small acervuli oozing conidia; culture in reverse pale greyish-brown with a greyish-salmon center. On

SNA 9 cm at seven days, mycelium uniform, low, white, opaque (Figure 2B); colorless in reverse; acervuli few, colorless. *Vegetative hyphae* 1.1-8.0 μm , average 3.2 μm . *Conidiomata* acervular. *Conidiogenous cells* hyaline, 4.3 - 18.2 \times 2.2 - 4.1 μm , ave. 11.3 \times 3.2 μm . *Conidia* hyaline, pale orange in mass, 1-celled, 6.4 - 16.0 \times 3.2 - 5.4 μm , ave. 10.9 \times 4.2 μm ($n = 41$), L/W ratio 2.6, cylindrical to ellipsoidal with ends broadly or acutely rounded (Figure 2C). *Appressoria* reniform, brown, smooth-walled, margin entire, 5.9 - 9.8 \times 3.9 - 7.7 μm (Figure 2F). *Setae* on OA rare, brown, thick-walled, irregular margin, tip acute, 68.5 - 73.1 \times 3.4 - 3.7 μm (Figure 2D and E).

Host: *Praelongorthezia praelonga*. Figure 2G-H.

Diagnosis: *Collectotrichum nymphaeae* var. *entomophilum* is distinguished morphologically from *C. nymphaeae* var. *nymphaeae* by its smaller conidia 6.4 - 16 \times 3.2 - 5.4 μm , ave. 10.9 \times 4.2 μm and the presence of setae (Table 5). The average length and width of conidia reported for

Table 5 – Colony characteristics on oatmeal agar (OA) and synthetic nutrient-poor agar medium (SNA) at seven days and morphological measurements of SNA cultures for *C. nymphaeae* var. *entomophilum* and *C. nymphaeae* var. *nymphaeae*. Information not provided is indicated by a dash (-).

<i>Colletotrichum nymphaeae</i>	Colony diameter (cm) 7 days		Conidia		Setae
	SNA	OA	Length [ave] (μm)	Width [ave] (μm)	
var. <i>entomophilum</i>	9.0	10.5	6.4-14.9 [10.9]	3.5-4.9 [4.2]	present
var. <i>nymphaeae</i> (CBS 515.78)	3.6	5.0	12.3-32.7 [17.2]	3.7-6.9 [5.0]	absent
<i>nymphaeae</i> (Damm et al., 2012)	1.65-2.6	1.45-2.9	10-19.5 [16.1]	3-6 [4.9]	absent
<i>nymphaeae</i> (Hemmi and Kawasi, 1954)	-	-	9-17 [-]	3-6 [-]	present

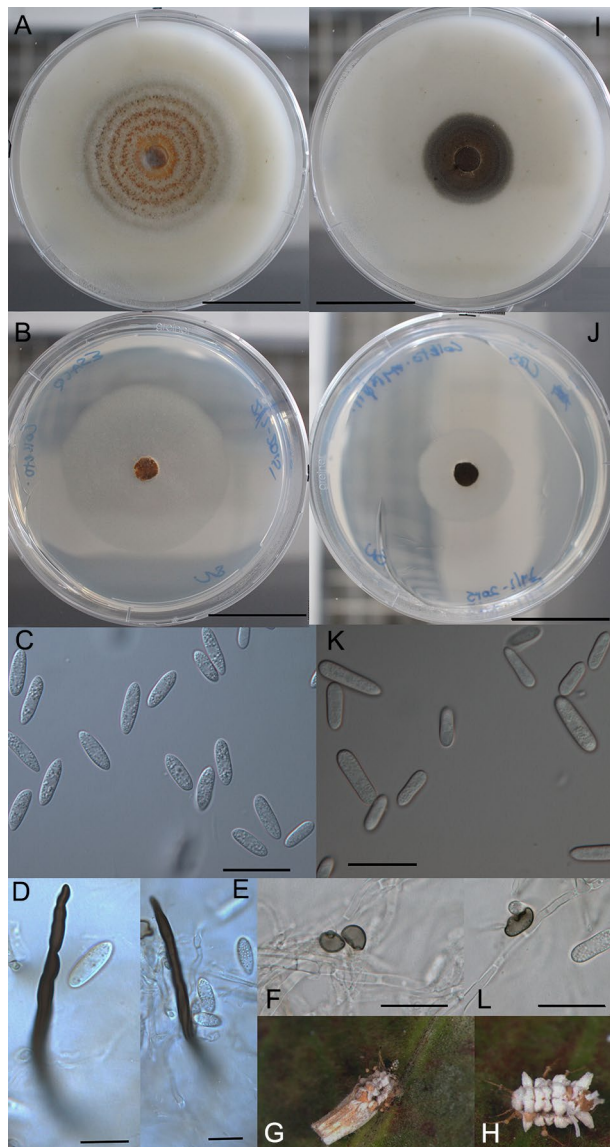


Figure 2 – A - H) *Colletotrichum nymphaeae* var. *entomophilum* (ESALQ 1393). A) culture on OA after eight days. B) culture on SNA after seven days. C) conidia. D - E) setae. F) appressorium. G - H) infected, sporulating, *Praelongortheziidae praelonga* cadavers. I - L) Plant pathogenic *C. nymphaeae* var. *nymphaeae* (CBS 515.78). I) culture on OA after seven days. J) culture on SNA after seven days. K) conidia. L) appressoria. Scale bars: A-J = 20 μm; C-F, K-L = 10 μm.

C. nymphaeae is $16.1 \times 4.9 \mu\text{m}$ with the exception of one strain, CBS 526.77, which was reported as having conidia measuring $9 - 13 \times 3 - 4.5 \mu\text{m}$ (Damm et al., 2012). In addition to morphological differences, *C. nymphaeae* var. *entomophilum* is distinct from all other isolates of *C. nymphaeae* by a single nucleotide mutation in the beta-tubulin gene (Table 3), two nucleotide mutations in the CHS-1 gene (Table 4) and by its insect, rather than plant host preference.

Additional notes. *Colletotrichum nymphaeae* var. *entomophilum* more closely matches the description of *Gloeosporium* (*Colletotrichum*) *nymphaeae* Hemmi and Kawase, isolated from leaves of the *Nymphaea* in Japan (Hemmi and Kawase, 1954), than the description of *C. nymphaeae sensu* Damm et al. (2012). Hemmi and Kawase report dark brown thick-walled setae, $19 - 76 \mu\text{m}$ long and conidia ranging from $9 - 17 \times 3 - 6 \mu\text{m}$. Unfortunately, the specimens cited by Hemmi and Kawasi (1954) cannot be found and no living cultures exist. According to Damm et al. (2012), *C. nymphaeae* as currently circumscribed is probably not the same organism described by Hemmi and Kawasi primarily because setae have never been observed in *C. nymphaeae*, neither by Damm et al. (2012) nor by van der Aa (1978); however, Batista and Bezerra (1966) described abundant setae in citrus orthozia insects infected by a "special strain" of *C. gloeosporioides*. Batista and Bezerra (1966) did not cite specimens in their study; however, the special strain they discovered and reported for the first time was likely *C. nymphaeae* var. *entomophilum*.

Additional specimens were examined at Cordeirópolis, São Paulo, 13 July 2005, *L.F. Padulla s.n.*, ESALQ 1368, and Rio de Janeiro, state of Rio de Janeiro, Feb 1994, *C.F. Robbs* ARSEF 4360.

Colletotrichum nymphaeae var. *nymphaeae*

(Pass.) Aa, Netherlands Journal of Plant Pathology, Supplement 1 84 (3): 110, Fig. 20. 1978; \equiv *Ascochyta nymphaeae* Pass., *Hedwigia* 16:120, 1877. **Lectotype**, Italy, Parma, from the leaf of *Nymphaea alba* (Nymphaeaceae), summer 1875, *G. Passerini*, 176820 (κ, non vidi); **Epitype**, Netherlands, Ubbergen, Oude Waal near Nijmegen, from the leaf spots of *Nymphaeae alba*, 7 Aug 1978, *G. van der Velde* (CBS H-20787), ex-epitype culture CBS 515.78! = van der Aa No. 657.

Colonies on OA 3.6 cm in seven days, a low felty mycelium with white, floccose center (Figure 2I-J); acer-

vuli salmon colored; culture in reverse dark brownish-grey. On SNA 5.0 mm in seven days, flat with a diffuse but well-defined irregular margin, hyaline to whitish; acervuli scattered, colorless. *Vegetative hyphae* 1.1 - 3.2 µm, septate, branched. *Conidiogenous cells* hyaline, 13.3 - 20.9 × 3.2 - 3.8 µm, ave. 17.1 × 3.6 µm. *Conidia* hyaline, pale orange in mass, 1-celled, 12.3 - 24 (-32.7) × 3.7 - 6.9 µm, ave. 17.2 × 5.0 µm (n = 31), L/W ratio 3.4, cylindrical, frequently narrowly obovate (Figure 2K). *Appressoria* reniform to elongate with crenulate margins, brown, 7.6 - 10.5 × 4.8 - 9.8 µm (Figure 2L). *Setae* not observed.

Discussion

Delineating taxa and resolving the relationships within *Colletotrichum* is a challenge because of high sequence homogeneity and morphological uniformity within the genus. For this reason, multiple genes are required not just for elucidating the relationships between taxa but also for species identification. Using DNA sequence data from five gene regions: GDPH+TUB2+ITS+ACT+CHS-1, we found strong support for including the insect pathogenic isolates of *C. nymphaeae* (formerly *C. gloeosporioides* f. sp. *ortheziidae* in the species *C. nymphaeae* (97 % bs) (Figure 1). On the basis of morphological and molecular characters we recognize the insect pathogenic isolates of *C. nymphaeae* as a new variety and designate the name *C. nymphaeae* var. *entomophilum*. A single nucleotide mutation in the GADPH gene, two mutations in CHS-1, and the presence of setae readily distinguishes this new variety from its closest relatives. Once additional informative markers are found for *Colletotrichum*, it may be possible to better resolve the relationships within *C. nymphaeae* and determine if *C. nymphaeae* var. *entomophilum* should continue to be recognized as a variety or as a distinct species. Until then, the circumscription of *C. nymphaeae sensu* Damm et al., 2012, should be modified to include setae. Setae are present in *C. nymphaeae* var. *entomophilum* but have so far not been observed in plant symbiont isolates of *C. nymphaeae*. In order to clarify their taxonomic utility, further studies should be conducted to confirm the presence of setae *in situ* and to elucidate any effects that sub-culturing or other environmental conditions might have on the production of setae *in vitro*.

Both entomopathogenic *Colletotrichum* taxa, *C. nymphaeae* var. *entomophilum* and *C. fiorinae*, display remarkably diverse lifestyle strategies with the ability to live as insect pathogens, plant pathogens and endophytes. At least 50 phytopathogenic isolates of *C. fiorinae* have been identified by DNA sequencing (Damm et al., 2012). Additionally, *C. fiorinae* has been isolated from fruit rot and detected as an endophyte in 28 species of plants in the region of epizootic outbreaks on the hemlock scale insect (Damm et al., 2012; Marcelino et al., 2009). *Colletotrichum nymphaeae* var. *entomophilum* has also been recovered as an endophyte but only in plants inoculated with the fungus (Marcelino et al., 2009). The

occurrence of *C. nymphaeae* var. *entomophilum* in nature, either as an endophyte or on additional insect hosts has not been studied. This is an area of research that merits prompt attention given that endophytic entomopathogens may serve as defensive mutualists when living in plant tissue (Bultman and Faeth, 2002; Crouch et al., 2014; Redman et al., 2002). For example, if *C. nymphaeae* var. *entomophilum* occurs endophytically in nature, it may potentially play a larger role in controlling citrus scale and other sap-sucking insects than previously realized.

The significance of the varied lifestyle strategies of *C. fiorinae* and *C. nymphaeae* var. *entomophilum* has previously been downplayed because the insect hosts of these fungi are plant sucking insects (Damm et al., 2012). A close relationship between insect pathogenic fungi and grass endosymbionts has previously been shown (Spatafora et al., 2007). Comparative genomic analyses showed that insect pathogenic *Metarhizium* spp. are more closely related to endophytes and plant pathogens compared to animal pathogens (Gao et al., 2011). The evolutionary transition from plant pathogen to endophyte in *Colletotrichum* is thought to occur relatively easily; for example, in at least one species of *Colletotrichum*, a single-locus mutation converts the fungus from a symptomatic plant pathogen to an endophyte. However, transitioning from plant pathogen or endophyte to a pathogen of plant sucking insects may represent a more significant and complex physiological shift than is assumed with genes co-opted, evolved or acquired by horizontal gene transfer from a plant-associated fungus (Barelli et al., 2016). Gene expression in *Colletotrichum* is highly dependent on plant signaling (Crouch et al., 2014; O'Connell et al., 2012). *In vitro* signaling has been shown to be markedly different from *in planta* signaling even from the point of spore germination to the ultimate necrotrophic phase characteristic of plant pathogens (O'Connell et al., 2012). Transcriptomic studies comparing gene expression of the entomopathogenic *Colletotrichum* taxa *in planta*, *in insecta* and *in vitro* may provide insight into the mechanisms behind inter-kingdom host shifts or the potential of a dual life style as insect pathogens and endophytes; in turn, these insights may help to determine how much or little weight should be given to host preferences for delimiting *Colletotrichum* taxa.

Much remains to be known about *C. nymphaeae* var. *entomophilum*. For example, how it is dispersed, its insect host-range, if it occurs endophytically both in nature and in citrus orchards and if endophytic growth affects the fitness of the citrus scale insect. Improved development of this important natural enemy should include studies on the below ground control of Ortheziidae scale insects since these insects also live in soil litter in humid habitats and feed on fungi, mosses and plant roots (Kondo et al., 2013). Entomopathogenic endophytes are well-known among the fungi, especially from the order Hypocreales e.g., *Acremonium*, *Beauveria*, *Clonostachys*, *Isaria*, *Lecanicillium*, *Verticillium* (Vega,

2008; Vega et al., 2008); however, few are aware that the common plant pathogenic genus *Colletotrichum* (Glomerellales) also contains endophytic entomopathogens. Clarifying the taxonomy of *C. nymphaeae* var. *entomophilum* may provide the impetus for further research on this overlooked entomopathogenic fungus. The assignment of a formal name along with morphological and molecular characterization provides a solid framework for facilitating the evaluation and approval of this important fungus for biological control.

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Authors' Contributions

Conceptualization: Wynns, A.A.; Delalibera, I. Data acquisition: Wynns, A.A.; Delalibera, I.; Jensen, A.A. Data analysis: Wynns, A.A. Design of methodology: Wynns, A.A.; Delalibera, I. Writing and editing: Wynns, A.A.; Delalibera, I.; Jensen, A.A.; Eilenberg, E.

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